Impact of AIP and inhibitory G protein alpha 2 proteins on clinical features of sporadic GH-secreting pituitary adenomas

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Abstract

Introduction: In sporadic acromegaly, downregulation of AIP protein of the adenomas associates with invasive tumor features and reduced responsiveness to somatostatin analogues. AIP is a regulator of G_{α_i} signaling, but it is not known how the biological function of the G_{α_i} pathway is controlled.

Aim: To study *GNAS* and *AIP* mutation status, AIP and $G_{\alpha_{i-2}}$ protein expressions, Ki-67 proliferation indices and clinical parameters in patients having primary surgery because of acromegaly at a single center between years 2000 and 2010.

Results: Sixty patients (F/M, 31/29), mean age 49 (median 50), mean follow-up 7.7 years (range 0.6–14.0) underwent primary surgery. Four adenoma specimens (6.8%) harbored an *AIP* and 21 (35.6%) an activating *GNAS* (*Gsp*+) mutation. Altogether 13/56 (23%) adenomas had low AIP protein levels, and 14/56 (25%) low $G_{\alpha_{i-2}}$ staining. In regression modeling, AIP expression associated with $G_{\alpha_{i-2}}$ ($P=2.33 \times 10^{-9}$) and lower Ki-67 ($P=0.04$). In pairwise comparison, low AIP protein predicted high GH at last follow-up (mean 7.7 years after surgery, $q=0.045$). Extent of treatments given for acromegaly associated with higher preoperative GH ($P=7.94 \times 10^{-4}$), KNOSP ($P=0.003$) and preoperative hypopituitarism ($P=0.03$) and remission at last follow-up with change in 3-month postoperative IGF1 ($P=2.07 \times 10^{-7}$).

Conclusions: We demonstrate, for the first time, that AIP protein expression associates with $G_{\alpha_{i-2}}$ protein intensities in sporadic somatotropinomas, suggesting a joint regulation on somatostatin signaling. Low AIP level associates with higher proliferative activity and predicts high GH concentrations after long-term follow-up. The *AIP* mutation rate of 6.8% is fairly high, reflecting the genetic composition of the Finnish population.

European Journal of
Endocrinology
(2017) **176**, 243–252

Introduction

Pituitary adenomas occur with an overall rate of one case in 1064 of the general population (1). Regardless of their benign nature, pituitary adenomas can cause severe health problems related to mass compression, hypopituitarism as well as hormonal hypersecretion.

The majority of pituitary adenomas arise in a sporadic setting and often the exact mechanisms of tumorigenesis

remain unknown. Recent next-generation sequencing studies have shown that the somatic landscape of pituitary tumors is relatively silent (2, 3). The primary somatic pathogenic alteration occurring in somatotropinomas is the stimulatory guanine nucleotide (GTP)-binding protein alpha (*GNAS*) mutation. The *GNAS* gene (*Gsp* oncogene) is mutated in approximately 35% of sporadic

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somatotropinomas leading to constitutive activation of cyclic adenosine monophosphate (cAMP) synthesis (4).

Approximately 5% of pituitary tumors occur in the context of hereditary tumor predisposition (5, 6). Germline mutations in the aryl hydrocarbon receptor-interacting protein (*AIP*) gene predispose to pituitary adenomas (7). *AIP* mutations are especially enriched in patients with positive family history of homogeneous somatotropinomas and/or a young age at onset, although a subset of patients display neither of these features (5).

Recently, it was discovered that *AIP* deficiency is associated with pituitary tumorigenesis through elevated intracellular cAMP levels (8, 9). In *AIP*-deficient cells, constitutive activation of cAMP production was caused by defective inhibitory Ga protein (G_{α_i}) signaling and $G_{\alpha_{i-2}}$ protein levels were markedly reduced in *AIP*-associated somatotropinomas (9). The G_{α_i} signaling pathway participates in the regulation of somatostatin by inhibiting growth hormone (GH) and prolactin (PRL) secretion and cAMP synthesis through somatostatin receptors (SSTR1–5) (10, 11). The results indicate that *AIP* acts as a regulator of G_{α_i} signaling, but it is not known how *AIP* controls the proper biological function of the G_{α_i} pathway.

Recent studies have indicated that downregulation of *AIP* protein contributes to the pathogenesis of sporadic pituitary adenomas, regardless of *AIP* mutation status. In patients with acromegaly, downregulation of *AIP* appears to predict tumor invasiveness and reduced response to postoperative somatostatin analogue (SSA) treatment. Moreover, it has been shown that preoperative SSA treatment upregulates *AIP* protein levels, further supporting the role of *AIP* in SSA signaling (12, 13, 14, 15).

To gain insight into the possible contribution of *AIP* and $G_{\alpha_{i-2}}$ protein expressions on therapeutic outcomes, we studied all consecutive patients with acromegaly ($n=60$) who underwent primary surgery at the Helsinki University Hospital (HUU) between years 2000 and 2010. We performed *AIP* and $G_{\alpha_{i-2}}$ immunohistochemical (IHC) semiquantitative analyses from the adenoma specimens, and related protein intensities to clinical data and treatment outcomes. We assessed the prevalence of *AIP* mutations and evaluated the possible influence of somatic *GNAS* mutation status on these variables.

Subjects and methods

Patient material

The patient cohort comprised 60 patients with acromegaly, who were operated on between years 2000 and 2010

at HUU (16, 17). Of the patients, 29 were males and 31 females with a mean age at surgery of 49 years (median 50). We retrospectively studied the corresponding 60 formalin-fixed paraffin-embedded (FFPE) pituitary adenoma samples (56 GH and 4 GH/PRL) from these patients. Clinical data were collected before and at 3, 6 and 12 months after surgery until the last follow-up. Mean follow-up time after surgery was 7.7 years (range 0.6–14.0). Most of the patients were primarily operated on; five patients received preoperative SSA treatment. All patients underwent pituitary magnetic resonance imaging (MRI) before surgery and at 3 months after surgery, after which follow-up imaging was scheduled on an individual basis.

Biochemical analysis

GH, prolactin (PRL) and insulin-like growth factor 1 (IGF1) measurements were obtained for each patient. Laboratory measurements were performed at HUSLAB, the laboratory of HUU, using standard accredited assays. Serum GH was determined with immunometric TR-IFMA and IGF1 with immunoenzymometric ELISA. IGF1 concentrations were expressed as percentage of upper limit of normal (%ULN). Serum prolactin was determined with immunometric TR-IFMA.

Hormonal remission and hypopituitarism

Biochemical control was defined according to the recent consensus recommendation of 2014 as basal serum GH concentrations $<1.0\mu\text{g/L}$ and normal age- and gender-standardized serum IGF1 concentrations, and for patients not on SSA treatment as suppression of serum GH concentration $<0.4\mu\text{g/L}$ on a two-hour oral glucose tolerance test (OGTT) (18). Hypopituitarism was assessed as previously described (17).

Data on the extent of given treatments were collected as an estimate of the aggressiveness of the disease. Different treatment combinations were grouped as follows: (1) patients treated with primary surgery only, (2) patients primarily operated on, treated with adjuvant medical therapy or fractionated stereotactic radiotherapy as well as adjuvant medical treatment or patients who were operated on twice. Group 2 included the tumors most resistant to treatment.

In addition, the treatment response and remission status were assessed by dividing IGF1 response into three groups as done previously (14): group 1, controlled

(IGF1 normalization for age and gender), group 2, partially controlled (non-normalized IGF1 with $\geq 30\%$ reduction compared to pretreatment values) and group 3, uncontrolled ($\leq 30\%$ decrease in IGF1 concentration or increase compared to pretreatment values).

Ethics

The study was approved by the review board of the Helsinki University Hospital.

DNA isolation and genetic screenings

DNA from FFPE tumors was isolated with NucleoSpin FFPE DNA isolation kit (Macherey-Nagel, Düren, Germany). Mutation screening of *AIP* was performed using PCR primers designed for FFPE-isolated DNA ([Supplementary Table 1](#), see section on [supplementary data](#) given at the end of this article). All six exons and exon–intron junctions were screened. Gain-of-function mutations of the *GNAS* gene at codons 201 and 227 (*Gsp* oncogene) were screened as previously described ([19](#)). The DNA fragments were amplified using AmpliTaqGold enzyme (Applied Biosystems) and purified using ExoSAP-IT PCR Purification Kit (USB Corporation, Cleveland, OH, USA). The sequencing reactions were performed utilizing the Big Dye Terminator v.3.1 Kit and ABI3730 Automatic DNA Sequencer (Applied Biosystems).

Immunohistochemistry

Antibodies used were mouse monoclonal antibody against AIP (clone35-2, Novus Biologicals LLC, Littleton, CO, USA; 1:500), Ga_{i-2} (T19, sc-7276, Santa Cruz, 1:70) and Ki-67 (MIB-1, M7240, Dako; 1:200). Anti-mouse/rabbit/rat secondary antibody, Poly-HRP-GAM/R/R (DPVB55HRP, Immunologic, Duiven, Netherlands) and DAB chromogen (Thermo Fisher Scientific) were used for detection. AIP protein expressions were semiquantitatively scored according to intensity (0, negative; 1, weak; 2, moderate; 3, strong) and expression pattern (1, patchy; 2, diffuse) as previously described ([14](#), [20](#), [21](#)). The final protein scores were calculated by multiplying intensity $\times$ expression pattern. Ga_{i-2} immunoreaction was scored in a similar way to AIP. Ki-67 scores were obtained by calculating the average percentage of stained nucleus among the cell population. Images were acquired with Leica DM LB microscope (Meyer Instruments, Houston, TX, USA), Olympus DP50 camera (Olympus Corporation) and Studio Lite software (Licor, Lincoln, NE, USA).

Statistical analyses

Statistical analysis was performed using R (version 3.0.2) and Python (version 2.7, packages NumPy, SciPy and StatsModels).

AIP and Ga_{i-2} levels were categorized into two groups according to the semiquantitative scoring (0–3: low; 4–6: high). Association of both AIP and Ga_{i-2} to a number of clinical variables was tested using the Mann–Whitney *U* test (SciPy function `mannwhitneyu`) similar to ([14](#)). Both nominal *P* values and the false discovery rate (FDR) adjusted *P* values (*q*-values) corrected with the Benjamini–Hochberg method were calculated.

In addition to pairwise tests, we performed linear regression modeling to account for the dependencies between clinical and genetic variables. A separate linear regression model was constructed to model AIP and Ga_{i-2} protein levels, as well as the extent of treatments, remission status and the change in pre- and post-treatment IGF1 concentrations (Δ IGF1, %ULN). An iterative procedure was used to find a linear model best predicting each of these variables, given clinical and genetic data. The set of explanatory variables considered for each model is described in [Supplementary Table 2](#).

In each of the iterations, the explanatory variable that yielded the highest increase in the R^2 adjusted for the number of model variables was added to the model. This procedure was initiated with a model containing no variables. To avoid overfitting while explaining as much variance in data as possible, the model with the lowest Bayesian information criterion (BIC) value was selected as the final model.

Results

AIP and *GNAS* mutations and clinical characteristics of the patient cohort

All 60 FFPE tumors were successfully sequenced and analyzed for *AIP* and *GNAS* mutations. An *AIP* mutation was identified in four tumors (4/59, 6.8%). A Finnish founder mutation, c.40C>T (Q14X), was found in three tumors and, in one tumor, a short out-of-frame deletion c.70>89delGAGCTCCCGGACTTTCAAGA (ENST00000279146) was observed. These mutations unambiguously resulting in a truncating transcript showed loss of the wild-type allele (LOH) as well ([Fig. 1A](#)). *AIP*mut+ patients were 18, 29, 35 and 42 years at diagnosis ([Table 1](#)). Gain-of-function *GNAS* mutations (*Gsp*+) were observed in 21/59 (35.6%) of tumors. Exon 8 mutation, c.1025C>T (R201C), was present in 17 tumors, and

Figure 1

(A) *AIP* exon 1 mutations found in sequenced somatotropinomas; a short out-of-the-frame c.70>89delGAGCTCCCGGACTTTCAAGA deletion (upper panel) and a Finnish Q14X nonsense mutation (lower panel) with LOH. The upper panel shows the wild-type (WT) and the mutant allele sequences. The deleted region is colored red in the WT sequence. The exact deletion breaking points were confirmed by sequencing the amplicon in both directions. (B) Immunostainings for AIP and Ga_{i-2} proteins in somatotropinomas. The upper panel shows diffuse cytoplasmic AIP immunoreactions and the lower panel cytoplasmic/membranous Ga_{i-2} stainings in corresponding pituitary tumors. 1=weak, 2=moderate and 3=strong immunoreaction intensity. *AIPmut+*=*AIP* mutation-positive somatotropinoma. Scale bar=10 μ m.

c.1025C>A (R201S) and c.1026G>A (R201H) mutations were both identified once. Exon 9 mutations, c.1104A>G (Q227R) and c.1104A>T (Q227L), were detected in one tumor each (ENST00000371085). *AIP* and *GNAS* mutations were mutually exclusive.

Characteristics of the 60 patients, grouped according to *GNAS* mutation positivity (*Gsp+*, $n=21$), wild-type ($n=35$) or *AIP* mutation positivity (*AIPmut+*, $n=4$) are given in Table 1. *AIPmut+* patients tended to be younger at diagnosis (31 years vs 52 and 49 years for *Gsp+* and wild-type tumors, respectively), were all characterized by

macroadenomas and, in 75% of cases, by a KNOSP grade ≥ 2 (Table 1). At three months after surgery, 67% ($n=40/58$) was in hormonal remission and at last follow-up, after a mean 7.7 (range 0.6–14.0) years 75% ($n=45/58$).

Fifty-four patients were operated on once and 6 patients underwent reoperation. Six patients received radiotherapy, and 20 patients received adjuvant medical therapy (SSA in 18 cases, cabergoline in 10 cases and pegvisomant in 3 cases) (Table 1).

Immunohistochemistry

AIP protein immunoreactivity was observed in 55 of 56 *AIPmut-* tumors (98%). Diffuse cytoplasmic staining was most prominent (Fig. 1B). Occasional nuclear immunoreactivity was also detected. Low AIP expression was observed in 12/56 (21%) of the tumors, one of which stained negative for AIP. Ga_{i-2} immunoreaction was positive in all adenomas, with prominent cytoplasmic and membranous staining. Low Ga_{i-2} protein expression (intensity ≤ 3) was found in 15/56 (27%) adenomas. Tumors with low AIP and Ga_{i-2} expression were largely overlapping. Ten tumors with low AIP level (intensity ≤ 3) also showed reduced Ga_{i-2} expression (Supplementary Fig. 1). Three *AIPmut+* tumors had enough tumor material for immunostainings (two Q14X and one tumor with c.70>89delGAGCTCCCGGACTTTCAAGA deletion). Two tumors showed negative AIP immunostaining and one tumor with Q14X mutation showed occasional cytoplasmic staining of single cells (Fig. 1B). Ga_{i-2} protein was expressed in all three *AIPmut+* somatotropinomas with reduced levels (intensity ≤ 2).

Relationship between AIP and Ga_{i-2} protein expression and clinical and hormonal variables

A strong positive association between AIP and Ga_{i-2} protein expression levels was observed (Mann–Whitney U test, $q=9.02 \times 10^{-5}$) (Table 2). AIP expression correlated inversely with last known GH ($q=0.045$). These correlations were the only significant pairwise associations to AIP levels with false discovery rate (FDR) $<10\%$ (Table 2 and Supplementary Tables 3 and 4). To analyze the relationship of other clinical parameters to AIP levels while controlling for Ga_{i-2} levels, linear regression was employed. A regression model consisting of Ga_{i-2} expression level, Ki-67 and three-month postoperative GH concentration was constructed, explaining 55% of the variance in AIP levels ($R^2=0.546$, $P=6.03 \times 10^{-9}$).

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Table 1 Clinical characteristics in 60 patients with acromegaly according to tumor type.

	Gsp⁺ (n=21)	Wildtype (n=35)	AIPmut⁺ (n=4)
Demographics			
Gender, M/F	8/13	18/17	3/1
Mean age at diagnosis (years) (s.d.)	52 (15)	49 (11)	31 (10)
Mean follow-up time (years) (s.d.)*	7 (3)	8 (3), 2 ND	11 (4)
Tumor characteristics			
Tumor size, micro (%) / macro (%)	10/90	14/86	0/100
Suprasellar extension, n (%)	6 (29)	21 (62), 1 ND	3 (75)
KNOSP grade 2 or above, n (%)	5 (24)	18 (55), 2 ND	3 (75)
Ki-67 <3%, n (%)	15 (75), 1 ND	29 (85), 1 ND	3 (100), 1 ND
Biochemical measurements at diagnosis			
Mean GH at diagnosis (μg/L) (s.d.)	56 (62), 14 missing	75 (85)	44 (36)
Mean IGF1 at diagnosis, xULN (s.d.)	2.5 (1.3)	2.1 (1.1), 3 ND	1.9 (0.9)
Mean PRL at diagnosis (U/L) (s.d.)	2900 (9500)	320 (290)	12 000 (24 000)
Hypopituitarism at diagnosis, n (%)	9 (43)	15 (43)	2 (50)
Treatment			
Reoperation, n (%)	1 (5)	4 (11)	1 (25)
Radiotherapy, n (%)	1 (5)	4 (11)	1 (25)
Any adjunctive medical therapy, n (%)	4 (20), 1 ND	13 (40), 3 ND	3 (75)
Somatostatin analogue, n (%)	4 (20), 1 ND	12 (38), 3 ND	1 (25)
Cabergoline, n (%)	2 (10), 1 ND	5 (16), 3 ND	3 (75)
Pegvisomant, n (%)	1 (5), 1 ND	2 (6), 3 ND	0 (0)
Preoperative medical treatment			
SSA, n (%)	1 (4.8)	4 (11.4)	0 (0)
Cabergoline, n (%)	3 (14.3)	0 (0)	0 (0)

*Time between diagnostic MRI and last clinical follow-up visit.

ND, samples with missing data percentages may not sum up to 100 because of rounding.

As expected from the strong pairwise association, most of the variance (43%) was explained by Ga_{i-2} level only (Ga_{i-2} regression coefficient $b=0.678$, 95% CIs (0.492, 0.867), $P=2.33 \times 10^{-9}$) (Table 3). In addition, the model was able to identify a weak correlation between higher AIP level and lower Ki-67 proliferation index ($b=-0.064$, 95% CIs (-0.126, -0.003), $P=0.04$). Also, three-month GH concentration improved the strength of the model, although the inverse correlation was not statistically significant (Table 3). An analogous regression model was obtained for Ga_{i-2} , consisting of the AIP level, Ki-67 scores and three-month postoperative GH concentration ($R^2=0.509$, $P=3.72 \times 10^{-8}$; Table 4).

The omission of five preoperatively SSA-treated patients from the data did not significantly alter statistical associations or correlations (data not shown).

Predictors of the extent of given treatments and remission status at the end of follow-up

Characteristics of patients treated with surgery only ($n=37$) compared to patients receiving any additional treatment ($n=20$) are given in Table 5. Patients who needed combined treatments were characterized by higher

preoperative GH concentration ($q=5.63 \times 10^{-4}$), higher KNOSP grade ($q=0.045$) and higher GH ($q=3.01 \times 10^{-5}$) and IGF1 ($q=1.24 \times 10^{-4}$) concentrations 3 months after surgery (Mann–Whitney U test). Hormonal remission at the end of follow-up was achieved in significantly fewer of the patients needing multimodal treatments compared to those who needed primary surgery only ($q=0.031$) (Table 5).

Regression modeling demonstrated that the extent of treatments given for acromegaly associated with more invasive tumor characteristics ($b=0.432$, 95% CI (0.161, 0.703), $P=0.003$), higher preoperative GH ($b=0.003$, 95% CIs (0.001, 0.004), $P=0.001$) and higher prevalence of preoperative hypopituitarism ($b=0.275$, 95% CIs (0.023, 0.527), $P=0.033$) (Supplementary Table 5). Inclusion of the preoperative IGF1 and the weight of the patient contributed to the model strength, although their model coefficients were not significant. This regression model explained 47% of the variance in the extent of treatments.

Low hormonal remission status at the last follow-up associated with poor IGF1 response three months after surgery, controlled for extent of treatments and sex (three-month Δ IGF1 change from Δ IGF1=0 to Δ IGF1=2,

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Table 2 Clinical characteristics of patients with sporadic somatotroph adenomas according to AIP expression status. AIP mutation-positive tumors are excluded from analysis.

	Total (n)	Low AIP expression (0–3)	High AIP expression (>3)	r	P	q
Demographics						
Gender, M/F	56	07/05/16	19/25	0.049	0.76	0.97
Mean age at diagnosis (years)	56	48.5	50.6	0.11	0.44	0.89
Mean follow-up time (years)*	54	7.8	7.4	0.048	0.74	0.96
Tumor characteristics						
Macroadenoma, n (%)	56	12 (100)	37 (84.1)	0.2	0.33	0.86
KNOSP grade 2 or above, n (%)	54	3 (25.0)	9 (20.5)	0.035	1	1
Mean Ki-67	54	2.1	1.4	0.2	0.14	0.62
Somatic GNAS mutation, n (%)	56	5 (41.7)	16 (36.4)	0.045	1	1
Ga _{i-2} protein level ≤3, n (%)	56	10 (83.3)	5 (11.4)	0.66	4.51 × 10⁻⁶***	9.02 × 10⁻⁵***
Biochemical measurements						
Mean GH at diagnosis (μg/L)	56	68.5	67.9	0.044	0.75	0.97
Mean IGF1 at diagnosis, ×ULN	53	2.2	2.3	0.042	0.77	0.97
Hypopituitarism at diagnosis, n (%)	56	5 (41.7)	19 (43.2)	0.012	1	1
Mean 3-month postoperative GH (μg/L)	54	15	7.6	0.24	0.076	0.4
Mean 3-month postoperative IGF1 (%ULN/100)	52	0.95	0.79	0.14	0.31	0.86
In remission at last follow-up visit (%)	56	75	88.1	0.15	0.36	0.87
Treatment						
Any kind of adjuvant treatment, n (%)	53	5 (41.7)	12 (27.3)	0.11	0.49	0.88
Radiotherapy, n (%)	56	0 (0)	5 (11.4)	0.16	0.35	0.87
Mean number of operations, n	56	1.2	1.1	0.14	0.57	0.91
Somatostatin analogue, n (%)	52	4 (33.3)	12 (27.3)	0.03	1	1
Cabergoline, n (%)	52	3 (25.0)	4 (9.1)	0.18	0.33	0.86
Pegvisomant, n (%)	52	0 (0)	3 (6.8)	0.13	0.58	0.91

Comparison by Mann–Whitney *U* test. Statistically significant values are displayed in bold.

****P* < 0.001. *Time between diagnostic MRI and last clinical follow-up visit.

r, effect size; *P*, *P* value; q, FDR-corrected *P* value.

$b = -0.776$, 95% CI $(-1.032, -0.520)$, $P = 2.07 \times 10^{-7}$) (Supplementary Table 6). A total of 65% of variance in the remission status was explained by the model.

A better IGF1 response at the last follow-up associated with less extensive treatments ($b = 0.418$, 95% CI $(0.132, 0.705)$, $P = 0.005$), lower preoperative prolactin level ($b = 1.19 \times 10^{-5}$, 95% CI $(5.36 \times 10^{-7}, 3.78 \times 10^{-5})$, $P = 0.044$) and slightly smaller preoperative GH concentration ($b = -0.003$, 95% CI $(-0.005, -0.001)$, $P = 0.014$) (Supplementary Table 7). This model explained only 21% of the total variance in IGF1 response.

Gsp mutation status and clinical characteristics

Gain-of-function *GNAS* mutation (*Gsp*+) was observed in 21/59 (35.6%) of the tumors. Association of *Gsp* mutation status to a number of clinical variables was tested using the Mann–Whitney *U* test. *Gsp* status had no effect on AIP or Ga_{i-2} protein levels (AIP $P = 1.0$; Ga_{i-2} $P = 1.0$; Supplementary Table 8). *Gsp* + tumors showed a nominal association with non-invasive characteristics (KNOSP (grades 0–2), $P = 0.02$, $q = 0.121$), as well as higher preoperative prolactin levels ($P = 0.02$, $q = 0.119$, median 203.0 ng/mL (*Gsp*–) and 528.0 ng/mL (*Gsp*)). However, after correcting for

Table 3 Linear regression model of AIP protein expression level.

Variable	b	SE	t	P	CI lower	CI upper
Constant	0.39	0.10	3.92	2.81 × 10⁻⁴***	0.19	0.59
Ga _{i-2}	0.68	0.09	7.33	2.33 × 10⁻⁹***	0.49	0.86
Ki-67	–0.06	0.03	–2.12	0.04*	–0.13	0.00
Three-month GH	–0.003	0.002	–1.07	0.29	–0.01	0.00

$n = 52$, degrees of freedom $df = 48$, adjusted $R^2 = 0.55$, $F = 21.4$, $BIC = 26.1$, $P = 6.03 \times 10^{-9}$ ***, b = coefficient, SE = standard error, t = t statistic, P = P -value, CI = 95% confidence intervals. The constant term b_0 of the regression model $y = b_0 + \sum b_i x_i + \varepsilon$ denoted by (Constant).

* $P < 0.05$ and *** $P < 0.001$.

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Table 4 Linear regression model of Ga_{i-2} protein expression level.

Variable	<i>b</i>	SE	<i>t</i>	<i>P</i>	CI lower	CI upper
Constant	0.08	0.12	0.68	0.50	-0.16	0.33
AIP	0.78	0.11	7.32	2.32 × 10⁻⁹***	0.57	0.99
Three-month GH	0.0003	0.003	0.10	0.92	-0.01	0.01
Ki-67	0.04	0.034	1.31	0.20	-0.02	0.11

n = 52, degrees of freedom *df* = 48, adjusted *R*² = 0.51, *F* = 18.6, BIC = 33.3, *P* = 3.72 × 10⁻⁸***, *b* = coefficient, SE = standard error, *t* = *t* statistic, *P* = *P*-value, CI = 95% confidence intervals. The constant term *b*₀ of the regression model $y = b_0 + \sum b_i x_i + \varepsilon$ denoted by (Constant).

****P* < 0.001. Statistically significant values are displayed in bold.

multiple testing, no significant associations with FDR < 10% were detected.

Discussion

We and others have previously reported that *AIP* germline mutations predispose to pituitary adenomas, mostly to somatotropinomas with an aggressive disease phenotype and a poor response to SSA treatment (5, 7, 15, 22). These recent data suggest that *AIP* is closely involved in SSA signaling and directly correlates with the therapeutic responses in the patients. In sporadic *AIP*mut-somatotropinomas, preoperative SSA treatment increases AIP protein expression and the AIP protein levels seem to predict the treatment responses to SSA therapy (12, 14, 21, 23). Recently, we demonstrated that *AIP* mutations predispose to pituitary tumorigenesis through defective inhibitory GTP-binding protein (Gai) signaling and that Ga_{i-2} protein levels were selectively reduced in *AIP*-mutated somatotropinomas (9).

Based on these findings, we wanted to examine the possible association between somatotropinoma AIP and Ga_{i-2} protein levels in patients with sporadic acromegaly, a majority of whom (93%) did not receive preoperative SSA treatment, and clinical, biochemical and imaging characteristics of the patients in our cohort of patients operated on at HUH during years 2000–2010 (16, 17). We found AIP and Ga_{i-2} protein expression levels to be strongly associated. Low AIP levels were seen together with reduced Ga_{i-2} protein expression, supporting the earlier finding where reduced Ga_{i-2} levels were detected in *AIP*-associated somatotropinomas (9). As downregulation of AIP is associated with reduced responsiveness to SSA treatment, and Ga_i proteins mediate somatostatin signaling via SSTRs (10, 11, 12, 14, 21), the simultaneous downregulation of AIP and Ga_{i-2} may reflect a joint regulation of these proteins on SSA signaling.

Albeit the majority of the AIP protein level variance was explained by Ga_{i-2}, also higher proliferative activity (Ki-67) and three-month postoperative GH concentrations

Table 5 Comparison between patients treated by primary surgery only and patients receiving multimodal treatments.

	Total (<i>n</i>)	Primary surgery only	Primary surgery + any other treatment modality	<i>r</i>	<i>P</i>	<i>Q</i>
F/M	53	18/18	9/8	0.03	1	1
Mean age at diagnosis (years)	53	51.4	47.8	0.11	0.43	0.89
Mean preoperative GH (μg/L)	53	42.7	129.8	0.54	3.76 × 10⁻⁵***	5.63 × 10⁻⁴***
Preoperative IGF1 (%ULN/100)	50	2.1	2.4	0.12	0.40	0.89
Size (micro/macro)	53	5/31	2/15	0.029	1	1
KNOSP grade >2, <i>n</i> (%)	53	4 (11.1)	8 (47.1)	0.40	0.006**	0.045*
Somatic GNAS mutation, <i>n</i> (%)	53	16 (44.4)	4 (23.5)	0.20	0.225	0.83
Ga _{i-2} protein level ≤3, <i>n</i> (%)	53	10 (27.8)	5 (29.4)	0.017	1	1
AIP protein level ≤3, <i>n</i> (%)	53	7 (19.4)	5 (29.4)	0.11	0.49	0.89
Mean Ki-67 proliferation index	51	1.6	1.6	0.029	0.84	1
Mean 3-month postoperative GH (μg/L)	52	3.4	22.3	0.63	7.53 × 10⁻⁷***	3.01 × 10⁻⁵***
Mean 3-month postoperative IGF1 (%ULN/100)	50	0.54	1.44	0.59	7.28 × 10⁻⁶***	1.25 × 10⁻⁴***
In remission at last follow-up visit (%)	52	97.1	64.7	0.44	0.003**	0.031*
Mean follow-up time (years)	52	7.1	8.8	0.28	0.044*	0.23

Comparison by Mann–Whitney *U* test. Statistically significant values are displayed in bold.

P* < 0.05, *P* < 0.01 and ****P* < 0.001.

r, effect size ($Z/\sqrt{N}$), where *Z* is the *Z* score corresponding to the *U* statistic and *N* is the total number of samples); *P*, Mann–Whitney *U* test *P* value; *q*, FDR-corrected *P* value.

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associated with reduced AIP protein expression. This supports earlier findings that *AIP*mut– somatotropinomas with reduced AIP levels share characteristics with *AIP*mut+ tumors. It was demonstrated that AIP downregulation is accompanied by higher proliferative activity and that *AIP* mutation-associated patients tend to have less effective reduction of GH and IGF1 levels, indicating poorer response to SSA treatments (14, 20, 22). In addition, it has been reported that low AIP level is a good marker of invasiveness in somatotropinomas (14, 21, 23). In the present study, however, no association between invasive features and AIP levels was detected. The reason for this difference might be the fact that in our tumor set, the total number of tumors having aggressive features was low (Table 1). However, low tumor AIP protein levels were significantly associated with higher GH concentrations at last follow-up, which occurred at a mean 7.7 years after surgery ($q=0.045$). This is, to the best of our knowledge, the first cohort study on sporadic acromegaly evaluating the possible influence of AIP protein levels of the primary tumors on long-term hormonal outcome, demonstrating that low AIP protein levels predict high GH concentrations even when more than 7 years after primary treatment have elapsed. In addition, regression modeling indicated that the extent of given treatments was best explained by the preoperative GH concentration ($P=7.94 \times 10^{-4}$), KNOSP ($P=0.003$) and preoperative hypopituitarism ($P=0.03$). Remission at the last follow-up was best explained by the change in 3-month postoperative IGF1 ($P=2.07 \times 10^{-7}$). Both low AIP protein level of the primary tumor, a small reduction only in IGF1 concentration 3 months after surgery, as well as preoperative GH, KNOSP grade and preoperative hypopituitarism thus seem to represent early and useful indicators of poor long-term hormonal remission and treatment resistance in sporadic acromegaly.

The current and previous studies suggest that downregulation of AIP protein contributes to the pathogenesis of a subset of *AIP*mut– somatotropinomas (12, 13, 14, 15, 21, 23). Currently, the mechanisms involved in the reduced AIP levels in these tumors are not known. Low somatic single-nucleotide and structural variant DNA damage rates in pituitary adenomas (2, 3) indicate the involvement of other mechanisms. It has been suggested that alterations in epigenetic regulation may play a prominent role in pituitary neoplasia, likely via loss or reduced expression of tumor suppressor genes (24). Also numerical chromosomal abnormality, aneuploidy and characterize these tumors (3, 25). Hence, these mechanisms may be promising candidates

in explaining reduced expression of AIP in these more aggressive *AIP*mut– somatotropinomas.

Although it has been proposed that *Gsp*+ associated tumors are more often microadenomas, lacking invasive features, and that patients with *Gsp*+ associated tumor respond better to SSA treatment, larger studies have shown that there is no difference in clinical features and outcome of patients with or without *Gsp* mutation (26, 27, 28). Consistent with this, we did not observe any correlation between adenomas with or without *Gsp* mutation and clinical characteristics. Further, our results agree with the study of Jaffrain-Rea *et al.* (14) in that no effect on AIP staining intensities was observed between *Gsp* genotypes. Although inhibitory G_{α_i} signaling counteracts the stimulatory action of GNAS (G_{α_s}) in cAMP synthesis, no correlation between *Gsp*+ genotype and $G_{\alpha_{i-2}}$ protein levels was detected.

Although AIP IHC is an important application in research use, the value of IHC for the detection of *AIP* germline mutation is more challenging. Many of *AIP* alterations are characterized by an exchange of single amino acid or in-frame insertion/deletion (indels) (5). In such cases, AIP IHC may lead to positive immunostaining, regardless of *AIP*-associated background of the tumor. Therefore, standard germline sequencing methods should be used for the identification of *AIP* germline mutations. For instance, multiplex ligation-dependent probe amplification (MLPA) and whole-genome sequencing (WGS) are methods of choice for identification of larger *AIP* germline indels.

In genetically heterogeneous populations, the prevalence of *AIP* mutations is generally less than 4% (20, 29, 30, 31, 32). In a Finnish setting, previous *AIP* mutation frequency studies have focused on a defined, homogenous population from Northern Finland or patients with early-onset disease (7, 29, 33). In the original study of Vierimaa *et al.* (7) *AIP* mutations accounted for 16% of population-based acromegaly patients from Northern Finland. In the current study, we examined the prevalence of *AIP* mutations in a cohort of 60 consecutive patients with sporadic acromegaly, operated at the HUH during 2000–2010, representative of the population in Southern Finland. Normal DNA samples were not available from the patients to perform germline *AIP* analysis. However, the appearance of a Finnish founder mutation, Q14X, supports the germline origin of this mutation. The *AIP* mutation prevalence of 6.7% seen in this apparently sporadic somatotropinoma setting is markedly higher than those reported in several prospective international cohort studies (5, 30, 31, 32).

This reflects the genetically homogenous structure of the Finnish population (34), where a Finnish AIP founder mutation (Q14X) accounts for the majority of the pathogenic variants (5, 7, 29, 33). In Finnish patients, we have so far identified altogether 29 Q14X mutations (21 familial and 8 apparently sporadic cases) and in addition, in patients with sporadic acromegaly, we have detected one splice-site mutation (IVS3-1G>A) (7) and two patients with short deletions: c.733>735delGAG (9) and c.70>89delGAGCTCCCGGACTTCAAGA (present study). The rareness of these variants compared to the Q14X mutation indicates that they represent more recent events in the Finnish population.

In summary, we have now for the first time shown that in sporadic somatotropinomas, AIP protein expression levels associate with $G_{\alpha_{i2}}$ protein intensities, possibly indicating a synergetic effect of these proteins on SSA signaling. In addition, higher proliferation activity and postoperative GH levels were seen together with reduced AIP protein levels, indicating a more aggressive nature of the tumors and worse treatment response. Both low AIP protein level of the primary tumor as well as a small reduction only in IGF1 concentration 3 months after surgery seem to serve as early and useful predictors of treatment resistance and poor long-term hormonal remission in sporadic acromegaly. In addition, the AIP mutation rate of 6.8% in our Finnish population-based series of somatotropinomas is higher than that seen in many other international cohort studies, likely reflecting the special genetic composition of the Finnish population.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/EJE-16-0620>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This study was supported by the Academy of Finland (grant number 250345, 2012–2017), the Cancer Society of Finland (grant number 4704019, 2015), Finska Läkaresällskapet, Novartis Finland and Pfizer Oy.

Author contribution statement

All authors reviewed the manuscript and approved the final version for publication. E Ritvonen is a principal investigator of the present study and wrote the manuscript together with E Pitkänen, A Karhu and C Schalin-Jäntti. E Pitkänen performed all statistical analyses. A Karppinen and S Vehkavaara participated in gathering the patient data. A Paetau was consulted about immunohistochemical analyses. H Demir participated

in genetic analyses. A Karhu and C Schalin-Jäntti were responsible for conceiving the idea of the present study and supervised the study.

Acknowledgements

The authors thank Sini Nieminen, Alison Ollikainen, Helena Ranta-aho, Inga-Lill Svedberg and Iina Vuoristo for technical assistance, and Nina Honkanen for collecting the paraffin samples. The Biomedicum Imaging Unit is acknowledged for the microscopy service.

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Received 26 July 2016

Revised version received 12 September 2016

Accepted 22 November 2016